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(54) Title: MODIFICATION OF LIGNIN SYNTHESIS IN PLANTS

(57) Abstract

The synthesis of lignin by plants is controlled by transformation of the plant genome with a recombinant gene construct which contains the gene specifying an enzyme critical to the synthesis of a lignin precursor, which gene may be in antisense orientation so that it is transcribed to mRNA having a sequence complementary to the equivalent mRNA transcribed from the endogenous gene thus leading to suppression of lignin synthesis. If the recombinant gene has the lignin enzyme gene in normal, or "sense" orientation, increased production of the enzyme may occur when the insert is the full length DNA but suppression may occur if only a partial sequence is employed.

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MODIFICATION OF LIGNIN SYNTHESIS IN PLANTS

This invention relates to the improvement of plants by the modfication of lignin biosynthesis, particularly, but not exclusively, the improvement of digestibility of fodder crops.

Grassland farmers, and farmers of other fodder crops, face a difficult decision each year about when to cut their crops for conservation. All grass varieties of agricultural importance suffer from the disadvantage that during the normal increase in dry matter yield with growth, the digestibility decreases. The farmer, therefore, has to compromise between a lower yield of highly digestible material and a higher yield of less digestible material. Another limitation is that harvesting at optimum maturity may be prevented by unfavourable weather. If the decline in digestibility could be controlled or delayed, higher yields of highly digestible material could be obtained and the prevailing weather conditions would not play such a major role in determining the quality of the harvested crop.

Digestibility of fodder crops is determined, among other factors, by the amount of lignification which has taken place during growth of the plants and the degree of secondary modification of lignin deposited. Beside cellulose and other polysaccharides, lignins are an essential component

of the cell wall in tissues like the sclerenchyma and the xylem of vascular plants. They play an important role in the conducting function of the xylem by reducing the permeability of the cell wall to water. They are also responsible for the rigidity of the cell wall, and, in woody tissues, they act as a bonding agent between cells, imparting to the plant a resistance towards impact, compression and bending. Finally, they are involved in mechanisms of resistance to pathogens by impeding the penetration or the propagation of the pathogenic agent.

Lignins are not only important in the productivity and performance of field crops but are also of great importance in trees for paper making. Considerable energy and chemical input is required to loosen, dissolve and remove lignin from the cellulose fibre which is required for paper making.

In addition to these instances in which lignins present a constraint on the use of crop plants, lignins are also used as feedstocks for the preparation of speciality chemicals such as phenolics which can be used as precursors in chemical synthesis. Thus lignins and their biological and chemical modification are important.

It is one of the objects of the present invention to provide a biotechnological procedure for the modification of both lignin content and lignin composition in plants.

Lignins are the product of a dehydrogenative polymerization of three primary precursors: the trans-coniferyl, trans-sinapyl and trans-p-coumaryl alcohols. The monomers can occur in lignins in different proportions and with different types of

linkages both with each other and with the surrounding cell wall polysaccharides,thus producing a wide variety of polymers. These polymers, or "lignin cores" are always associated covalently with hemicelluloses. Most lignins also contain varying amounts of aromatic carboxylic acids in ester-like combinations. Such differences in the structure of lignins are usually found in plant species. However, differences in the composition of lignins, and even in the binding to the primary and secondary cell walls, can also occur in the same plant, between different tissues of different ages. The biosynthesis of lignin monomers is a part of the phenylpropanoid biosynthesis pathway, which is also responsible for the production of a wide range of compounds including flavonoid pigments, isoflavonoids, coumarin phytoalexins and cell division promoting dehydrodiconiferyl glucosides.

Phenylalanine is deaminated to produce cinnamic acid. This acid is then hydroxylated and methylated, producing different acids substituted on the aromatic ring. Coenzyme A thioesters of (p)-coumaric, ferulic and sinapic acids are then produced by the action of hydroxycinnamate: CoA ligase. These compounds are subsequently reduced by cinnamyl-CoA reductase (CCR) to cinnamaldehydes, which are finally converted to cinnamyl alcohols by the cinnamyl alcohol dehydrogenase (CAD). Only the last two reactions are specific for the biosynthesis of lignin. The cinnamyl alcohols are then believed to be transported to the cell wall where they are polymerised by peroxidase in the presence of hydrogen peroxide.

When the surface growth of the c ll c ases, it is follow d by a phase of wall thickening (secondary wall formation). Lignification takes place predominantly during this phase. It starts in the cell corners and extends along the middle lamella, through the primary wall and, finally, to the secondary wall. External factors can induce qualititave and quantitative modifications in lignification. The synthesis of new types of lignins, sometimes in tissues which are not normally lignified, may be induced by infection with pathogenic microorganisms. Lignification is stimulated by light, as well as by low calcium levels, by boron, by mechanical stress and by infection.

Cinnamyl alcohol dehydrogenase: (CAD, E.C. 1.1.1.195) catalyses the conversion of cinnamaldehydes to cinnamyl alcohols. CAD has been characterised for several different species: Forsythia suspensa, soybean (Glycine max), spruce (Picea abies), poplar (Populus euramericana) and eucalyptus.

In most instances, only one form of CAD, has been detected for each species except for soybean which has two isoenzymes, one of 43,000 daltons and one of 69,000 daltons. The first soybean isoenzyme is specific for coniferyl alcohol while the 69,000 daltons soybean-isoenzyme and all other CAD can catalyse the formation of all the cinnamyl alcohols (i.e. coniferyl, sinapyl and coumaryl alcohols). However, the Km of CAD for the different cinnamyl alcohols varies between enzymes from different species. This variation may explain the different compositions of lignin core in differ nt species.

Indeed, lignin monomers cannot be synthesised in plants by any biochemical pathway not involving CAD and CCR. Thus CAD, as well as CCR, may be key enzymes in the regulation of lignification. The utilisation of inhibitors specific for these enzymes indicates that they may regulate the quantity of lignin rather than its composition. However, the Km values of soybean CAD isoenzymes for the different cinnamyl alcohols suggest that CAD isoenzymes may control the composition of lignin in some species. The presence of $Zn2+$ is required for the activity of CAD, as for other alcohol dehydrogenases. The reduction of cinnamaldehydes cannot be catalysed by CAD in the presence of NAD instead of NADP. The common sub-unit structure of CAD seems to be a dimer of approximately 80,000 daltons (each monomer having a molecular weight of approximately 40,000). However, it has been reported that the bean enzyme is a monomer with a molecular weight of 65,000, based on analysis of cDNA clones. Treatment of bean cell suspension cultures with a high-molecular-mass elicitor preparation heat-released from mycelial cell wall of the bean pathogen <u>Collectotrichum lindemuthianum</u> increases the extractable activity of CAD. The increased CAD activity might be regarded as a reaction of defense against pathogenic microorganisms, since an increase in the activity of this enzyme may be related to the deposition of lignin in the cell wall of infected cells, or to the synthesis of extracellular lignin-like material and other phenolic compounds involved in defense responses.

Walter et al. (1988) have constructed a lamda

gt11 cDNA library from elicitor-treated bean cells. This library was screened with antibodies raised against poplar CAD enzyme to identify the CAD cDNA clones. A 1.2 kb long cDNA clone was isolated and designated clone 4a. Subsequent experiments have however demonstrated that this clone does not encode CAD but malic enzyme (Walter et al., 1990).

Therefore, although it was known that CAD may be a useful target for the modification of crop plants, this was in fact not practically possible using the information available. The work leading to the present invention provides a new method for the isolation of CAD enzyme to homogeneity, and CAD cDNA clones from various species, which can now be used to modify lignification of crop plants.

Thus, plants with a reduced amount of lignin or modified lignin composition would be more efficiently used as a forage for cattle. The yield of milk and meat would be therefore increased. Furthermore, lignin may have a negative effect on plant growth. Thus, a reduction of the lignification in crops such as wheat, oilseed rape, sugar beet or maize might presumably increase the grain yield. Trees with reduced lignin contents or altered lignin structure will lead to a reduction in the cost of the paper as less lignin will have to be removed during the pulping process. On the other hand, novel papers may be produced due to the purity of cellulose fibre which could otherwise not be produced.

The principal applications of the present invention are improvement of the digestibility of forage crops, reduction of lignin in woody feedstocks for cellulose fibre extraction,

improvement of the response of crop plants to pathogen attack, and, improvement of timber quality. Some of these applications may require that the total amount of lignin be reduced: others may require that the amount of lignin be increased. It may also be the case that alteration of the chemical composition of the lignin polymer will confer advantages in the selected application.

Industrial processes for the extraction of cellulosic fibres from woody feedstocks amount in essence to a chemical extraction procedure for removing lignin. Once lignin is removed from the feedstock the cellulosic fibres may be recovered and manufactured into paper or utilised in other ways, for example the cellulose may be further processed into cellulosic films or yarn for weaving or knitting into fabrics. Reduction of the lignin synthesised by the plants used as feedstock, trees usually, will have a direct effect of reducing the chemical and energy demands of such extractive processes and reduce the amount of effluent material which well-recognised as a major potential environmental pollutant which is both difficult and expensive to process. Alteration of the chemical composition of the lignin will potentially alter the solubility characteristics of the lignin in the chemical extractants used. Again this should lead directly to a reduced usage of chemicals and lower energy requirements. Finally alteration of the lignin quality of presently unsuitable species may provide alternative feedstocks for the papermaking industry and the cut timber industry.

Reduction of lignification can be achiev d by the application of chemical inhibitors to plants.

However, a more effective method controlling lignin d position and structure is the inhibition of expression of the CAD gene using antisense RNA. Antisense RNA technology is an appropriate molecular biology approach to the inhibition of lignification. An antisense RNA is an RNA produced by the transcription of the non-coding DNA strand (nonsense). Thus, antisense RNA has the same sequence as the coding DNA strand and is complementary to the mRNA product of a specific gene.

As is well known, a cell manufactures protein by transcribing the DNA of the gene for that protein to produce RNA, which is then processed (e.g. by the removal of introns) into messenger RNA and finally translated by ribosomes into protein. This process may be inhibited by the presence in the cell of "antisense RNA". Therefore, as used herein, the term "antisense RNA" means an RNA sequence which is complementary to a sequence of bases in a mRNA: complementary in the sense that each base (or a majority of bases) in the antisense sequence (read in the 3′ to 5′ sense) is capable of pairing with the corresponding base (G with C, A with U) in the mRNA sequence read in the 5′ to 3′ sense. It is believed that this inhibition takes place by formation of a complex between the two complementary strands of RNA, preventing the formation of protein. How this works is uncertain: the complex may interfere with further transcription, processing, transport or translation, or lead to degradation of the mRNA, or have more than one of these effects. Such antisense RNA may be produced in the cell by transformation with an

appropriate DNA construct arranged to transcribe backwards part of the coding strand (as opposed to the template strand) of the relevant gene (or of a DNA sequence showing substantial homology therewith).

The use of this technology to downregulate the expression of specific plant genes has been described, for example in European Patent Publication No 271988 to ICI. Reduction of gene expression has led to a change in the phenotype of the plant: either at the level of gross visible phenotypic difference e.g. lack of lycopene synthesis in the fruit of tomato leading to the production of yellow rather than red fruit or at a more subtle biochemical level e.g. change in the amount of polygalacturonase and reduction in depolymerisation of pectins during tomato fruit ripening (Smith et al, Nature, 334, 724-726, 1988; Smith et al, Plant Mol Biol 14, 369-380, 1990). Thus antisense RNA has been proven to be useful in achieving down-regulation of gene expression in plants.

An object of the present invention is to provide plants having an altered ability to synthesise lignin.

According to the present invention there is provided a recombinant DNA comprising a plant DNA having, in sequence a gene promoter sequence a coding region and a gene terminator, said coding region comprising a nucleotide sequence encoding a mRNA which is substantially homologous or complementary to mRNA encoded by an endogenous plant gene or a part thereof which encodes an enzyme essential to lignin biosynthesis, so that,

when incorporat d into a plant genome by transformation, mRNA transcribed from the said coding region inhibits production of the enzyme from the endogenous gene.

Preferably the coding region encodes mRNA in antisense orientation to the mRNA encoded by the said endogenous gene. Such an antisense sequence may be isolated from the untranscribed strand of the DNA encoding the said endogenous gene.

However, the coding region may alternatively be in the same orientation as the said endogenous gene. Such construction may lead to overproduction of the endogenous enzyme or it may inhibit production of same.

It is preferred that the coding region has a minimum size of 50 bases.

The target enzyme for controlling lignin production may be selected from the group consisting of cinnamyl alcohol dehydrogenase (CAD), cinnamoyl: CoA reductase (CCR) and catechol-O-methyl transferase (COMT).

The promoter may be selected from promoters known to operate in plants but is preferably selected from the group consisting of CaMV35S, GPAL2, GPAL3 and endogenous plant promoter controlling expression of the endogenous target enzyme, for example, the promoter of the CAD gene.

The invention also provides a method of inhibiting or altering lignin biosynthesis in a plant, comprising stably incorporating into the genome of the plant by transformation a recombinant DNA comprising a plant DNA having, in sequence a gene promoter sequence a coding region and a gene terminator, said coding region comprising a

nucleotide sequence encoding a mRNA which is substantially homologous or complementary to mRNA encoded by an endogenous plant gene or a part thereof which encodes an enzyme essential to lignin biosynthesis, so that, when incorporated into a plant genome by transformation, mRNA transcribed from the said coding region inhibits production of the enzyme from the endogenous gene.

Further, the invention provides a transformed plant possessing lower than normal ability to produce lignin characterised in that said plant has stably incorporated within its genome a recombinant DNA as described hereinabove. Examples of plants which may be so transformed are maize, eucalyptus, aspen, poplar, and tobacco. However, the invention is not restricted to these crops and it is envisaged that suitable primary applications will be in forage crops such as alfalfa, lolium and festuca. However, control of lignin synthesis has wide potential application in many crops.

The invention also provides the following sources of suitable genes for use in construction the recombinant DNAs:

(i) Plasmids pTCAD14 or pTCAD19 (tobacco CAD) which have been deposited, in E.coli strain XL1Blue host, at the National Collection of Industrial and Marine Bacteria, Aberdeen, United Kingdom, under the Accession Number 40404, on 17th April 1991 and 40401 on 8th April 1991 respectively.

(ii) Plasmid pZCAD1 (maize CAD) which has been deposited, in E.coli strain XL1Blue host, on 2nd April 1992 at the National Collection of Industrial and Marine Bacteria, Aberde n, United Kingdom, under the Accession Number 40501.

(iii) Plasmid pPOPCAD1 (poplar CAD) which has been deposited, in E.coli strain XL1Blue host, on 2nd April 1992 at the National Collection of Industrial and Marine Bacteria, Aberdeen, United Kingdom, under the Accession Number 40500.

(iv) Plasmid pEUCAD1 which has been deposited, in E.coli strain XL1Blue host, on 2nd April 1992 at the National Collection of Industrial and Marine Bacteria, Aberdeen, United Kingdom, under the Accession Number 40502.

These plasmids have been deposited under the provisions of the Budapest Treaty on the Deposit of Microorganisms for Patent Purposes.

Thus, the invention includes the DNA insert contained in the clones pTCAD14, pTCAD19, pZCAD1, pPOPCAD1 and pEUCAD1 and variants thereof such as are permitted by the degeneracy of the genetic code or the functional equivalents thereof. In addition, the present invention provides a recombinant DNA construct containing the said DNA under control of a transcriptional control sequence operative in plants, so that the construct can generate mRNA in plant cells which can either be full-length or partial length in respect to the normal mRNA.

For the down-regulation of lignin synthesis the aforesaid DNA is in antisense or 'sense' orientation.

For the amplification of lignin biosynthesis the aforesaid DNA is in sense orientation thus to provide one or more additional copies of the said DNA in the plant genome. In this case the DNA is a full-length cDNA copy.

Thus, in a further aspect, the present

invention provides DNA constructs comprising a transcriptional initiation region operative in plants positioned for transcription of a DNA sequence encoding RNA complementary to a substantial run of bases showing substantial homology to an mRNA encoding the protein produced by the gene in pTCAD14, pTCAD19, pZCAD1, pPOPCAD1 and pEUCAD1.

The invention further provides plant cells, and plants derived therefrom having stably incorporated in their genomes the aforesaid DNA in sense or antisense orientation, and fruit and seeds of such plants.

The present invention is principally concerned with the suppression of lignin formation and, that being so, the inserted gene will be in antisense orientation, but there are instances where over-production of lignin may have an advantageous effect, for example to improve plant stalk strength, reduce plant stature and consequent lodging, and resistance to diseases, and the present invention provides means for achieving amplification of the lignin biosynthetic ability of plants.

Thus the invention relates generally to the regulation of the plant's lignin biosynthetic pathway, in which CAD plays a dominant role, in order that the production of CAD, and hence the production of lignin, may be increased, by supplying extra copies of the CAD gene which is the subject of this invention, or decreased by insertion of the CAD gene, or a portion thereof (usually of 50 or more bases), in antisense orientation so that the amount of CAD for

catalysing lignin synthesis is reduced.

The constructs of the invention may be inserted into plants to regulate the production of the CAD enzyme. Depending on the nature of the construct, the production of the protein may be increased, or reduced, either throughout or at particular stages in the life of the plant. It is also possible to target the expression of the gene to specific cell types of the plant, such as the epidermis, the xylem, the roots etc.

The plants to which the present invention can be applied include commercially important food and forage plants, such as alfalfa, maize, oil seed rape, forage grasses and sunflower, and also tree crops such as eucalyptus, pine species and poplar.

DNA constructs according to the invention preferably comprise a sequence of at least 50 bases which is homologous to the DNA of the insert in pTCAD19 or pTCAD14. There is no theoretical upper limit to the base sequence - it may be as long as the relevant mRNA produced by the cell - but for convenience it will generally be found suitable to use sequences between 100 and 1000 bases in length. The preparation of such constructs is described in more detail below.

The preferred source of antisense RNA for use in the present invention is DNA derived from the clones pTCAD19 and pTCAD14. The required DNA encoding antisense RNA can be obtained in several ways: by cutting an appropriate sequence of DNA from pTCAD19 or pTCAD14 (or any other source of the CAD gene); by synthesising a DNA fragment using synthetic oligonucleotides which are annealed and then ligated togeth r in such a way as to giv

suitable restriction sites at each end; by using synthetic oligonucleotides in a polymerase chain reaction (PCR) to generate the required fragment with suitable restriction sites at each end. The DNA is then cloned into a vector containing upstream promoter and downstream terminator sequences, the cloning being carried out so that the DNA sequence is inverted with respect to its orientation to the promoter in the strand from which it was cut. In the new vector, the strand that was formerly the template strand becomes the coding strand, and vice versa. The new vector will thus encode RNA in a base sequence which is complementary to the sequence of pTCAD19 and pTCAD14 mRNAs. Thus the two RNA strands are complementary not only in their base sequence but also in their orientations (5′ to 3′).

As source of the DNA base sequence for transcription, it is convenient to use a cDNA clone such as pTCAD19 and pTCAD14. The base sequence of pTCAD19 is set out in Figure 3 and the sequence of pTCAD14 is shown in Figure 4.

A source of DNA for the base sequence for transcription is the promoter of the CAD gene itself or other genes involved in lignification such as the promoter of the phenylalanine ammonia lyase gene or its modified version which permits expression in xylem tissue. Such a gene may differ from the cDNA of pTCAD19 or pTCAD14 in that introns may be present. The introns are not transcribed into mRNA (or, if so transcribed, are subsequently cut out). When using such a gene as the source of th base s quenc for transcription it is possible to use either intron or exon regions.

A further way of obtaining a suitable DNA base sequence for transcription is to synthesise it ab initio from the appropriate bases. Recombinant DNA and vectors according to the present invention may be made as follows. A suitable vector containing the desired base sequence for transcription (for example pTCAD19) is treated with restriction enzymes to cut out the sequence. The DNA strand so obtained is cloned (in reverse orientation) into a second vector containing the desired promoter sequence (for example cauliflower mosaic virus 35S RNA promoter or the bean PAL promoter, Bevan et al, EMBO J.8, 1899-1906 1988) and the desired terminator sequence (for example the 3' of the <u>Agrobacterium</u> <u>tumefaciens</u> nopaline synthase gene.

According to the invention we propose to use both constitutive promoters (such as cauliflower mosaic virus 35S RNA) and inducible or developmentally regulated promoters (such as the PAL gene promoter or the endogenous CAD gene promoter) as circumstances require. Use of a constitutive promoter will tend to affect functions in all parts of the plant: while by using a tissue specific promoter, functions may be controlled more selectively. The use of a tissue-specific promoter, has the advantage that the antisense or sense RNA is only produced in the tissue in which its action is required.

Vectors according to the invention may be used to transform plants as desired, to make plants according to the invention. Dicotyledonous plants, such as alfalfa, oil seed rape etc, may be transformed by <u>Agrobacterium</u> Ti plasmid technology, for example as describ d by Bevan (1984) Nucleic

Acid Research, 12, 8711-8721. Such transformed plants may be replicat d sexually, or by cell or tissue culture.

The degree of production of RNA in the plant cells can be controlled by suitable choice of promoter sequences, or by selecting the number of copies, or the site of integration, of the DNA sequences according to the invention that are introduced into the plant genome. In this way it may be possible to modify lignification to a greater or lesser extent.

The constructs of our invention may be used to transform cells of both monocotyledonous and dicotyledonous plants in various ways known to the art. In many cases such plant cells (particularly when they are cells of dicotyledonous plants) may be cultured to regenerate whole plants which subsequently reproduce to give successive generations of genetically modified plants. Examples of genetically modified plants according to the present invention include, alfalfa, oil seed rape, sunflower, sorghum, maize, festuca,and trees such as eucalyptus, poplar, and pine.

In the present invention, we use antisense RNA in order to determine the phenotype of transgenic plants which show modified, that is increased or reduced, expression of pTCAD19 or pTCAD14 by the use of antisense and sense expression vectors.

The invention will now be described further with reference to the accompanying drawings, in which:

Figure 1 shows the partial amino acid sequence determined from purified tobacco CAD protein,

Figure 2 shows the design of the oligonucleotide sequ nce used to identify a CAD clone,

Figure 3 shows the complete sequence of pTCAD19,

Figure 4 shows the sequence of pTCAD14 tobacco cDNA clone,

Figure 5 shows the construction of antisense and sense vectors using the EcoR1-HindIII fragment of pTCAD19/pTCAD14,

Figure 6 shows the construction of expression vectors containing the complete tobacco CAD cDNA clones,

Figure 7 shows the complete sequence of pPOPCAD1,

Figure 8 shows the complete sequence of pEUCAD1,

Figure 9 shows sequence of primers used to generate a maize CAD clone by PCR, and,

Figure 10 shows the sequence of pZCAD1, a 200bp PCR product from maize genomic DNA.

Figure 11 shows CAD activities of the control and antisense plants.

The invention will now be described, by way of illustration, in the following Examples.

Example 1

Development of an efficient purification protocol for CAD enzyme

Improved protocols have been developed for the purification of CAD. The new procedure is simpler than the previously published methods and is based on the following steps:

1. Preparation of tobacco stem xtract by homogenization and 70-40% ammonium sulphate precipitation.

Six week old tobacco stems were frozen in liquid N, crushed with a hammer and homogenized in a Waring blender in buffer A. The homogenate was centrifuged at 45000xg for 30min. Solid ammonium sulphate was added to the supernatant to bring it to 70% saturation and proteins were precipitated at 4°C for 30min. The precipitate was collected by centrifugation at 10,000rpm for 1 hour. The pellet was resuspended in a minimum volume of buffer supplemented with 5% ethylene glycol, to reduce the ammonium sulphate concentration to approximately 40% saturation. Material that did not resuspend was removed by centrifugation.

2. Affinity chromatography on Blue Sepharose.

The supernatant was desalted and applied to a Blue Sepharose column. The column was washed in at least 6 column volumes buffer including one column volume supplemented with 4mM NAD. This wash elutes other alcohol dehydrogenases. Specific elution of CAD was performed with a gradient of 0-4mM NADP in buffer B. CAD-containing fractions were pooled and 5% ethylene glycol was added.

3. Ion exchange FPLC on Mono Q.

The pooled fractions from Blue Sepharose were applied to an FPLC Mono Q column. The column was washed until the Absorbance dropped to baseline levels. Proteins were eluted in a linear gradient of buffer with 20-400mM Tris-HCl, pH 7.5.

4. Affinity chromatography on 2'5' ADP Sepharose.

MonoQ fractions were applied to a column of 2'5'ADP-Sepharose. The column was washed with 6

column volumes buffer including one column volume supplemented with 4mM NAD. Sp cific elution was performed in a linear gradient of buffer with 0-4mM NADP.

Using this protocol tobacco CAD was purified to homogeneity. 600μg were obtained from 4Kg material representing 0.05% total soluble protein. This represents a purification of approximately 2000 fold. The purified enzyme has a specific activity of 173nKat/mg protein. The pure enzyme is specific for NADP and exhibits a Km for coniferyl alcohol of 12μmol/l.

Example 2

Characterisation of CAD enzyme

Purified CAD consists of two subunits of approximate molecular weights 42.5kDa and 44kDa. When slices isolated separately from native gels containing the CAD protein (identified as two separate bands by silver staining) were transferred to and run on an SDS gel, each native form appears to contain both polypeptides. Pure protein was run on a reverse phase HPLC column, yielding two well separated protein peaks probably the two polypetides. Peptide mapping of each polypeptide with N- chlorosuccinimide/urea and amino acid analysis of purified subunits suggest that they are very similar.

Both peptides were digested with trypsin and the sequence of the resulting fragments was determined. The sequence of the peptides is shown in Figure 1.

This shows clearly that CAD is represented by two closely related polypeptid s.

Example 3

Establishment of a stem-specific cDNA library from tobacco

A cDNA library has been generated using RNA extracted from six week old tobacco stems. 20ug of polyA RNA was prepared and cDNA synthesised. Part of this was cloned into lambda-ZAP II vector (a commercially available cloning vector). This yielded 860,000 recombinants, 70% of which have inserts of 1Kb or greater, as determined by PCR on 24 randomly selected clones.

Example 4

Identification of a CAD cDNA

600,000 recombinants were screened using an oligonucleotide probe (CAD116) shown in Figure 2. This oligo was designed against peptide sequence 4 from Figure 1.

One strongly hybridizing clone was identified, purified and characterised. This clone, pTCAD19, has a cDNA insert of 1419 bp. Analysis of the DNA sequence-derived amino acid sequence clearly demonstrates that it represents a CAD clone as several regions show DNA sequence-derived amino acid sequences identical to the peptide sequences found in Figure 1 representing the 42.5 kDa peptide (peak 2 from RHPLC).

Example 5

Rescreening of the tobacco cDNA library with the insert of pTCAD19.

The 600,000 clones representing the tobacco stem cDNA library was rescreened using the EcoR1 insert of pTCAD19. Five additional clones were identified, purified and characterised.

Sequencing of these clones has allowed

identification of two cDNA clones which are different from pTCAD19, encoding the p ptid found in peak 1 from RHPLC. A representative clone is called pTCAD14 and its sequence is shown in Figure 5. This demonstrates that this clone is different from pTCAD19 and that it contains peptide sequence derived from the peak 1 protein.

Example 6

Generation of CAD antisense vectors

A. VECTORS BASED ON pJR1

pTCAD19 and pTCAD14 were cut with EcoR1 and HindIII, and the resulting fragments of 981 bases were isolated by agarose gel electrophoresis. The fragment was made blunt ended using Klenow fragment A. The fragment was then cloned into pJR1 cut with Sma1. Clones containing the insert in the antisense orientation are called pJT19A and pJT14A. Clones which contain the fragment in sense orientation are called pJT19S and pJT19S. The construction of these vectors is shown in Figure 5.

B. VECTORS BASED ON pMK4

pTCAD19 was cut with EcoR1 and HindIII, and the resulting fragment of 981 bases was isolated by agarose gel electrophoresis. The fragment was made blunt ended using Klenow fragment A. The fragment was then cloned into pMK4 cut with HincII. pMK4 is an expression vector which contains the Dral fragment of the bean phenylalanine ammonia lyase promoter, a multiple cloning site and the nos 3′ end. Clones containing the insert in the antisense orientation are called pMT19A and pMT14A . Clones which contain the fragment in sense orientation are called pMT19S and pMT14S. The construction of th se vectors is shown in Figur 5.

Example 7

Generation of CAD expression vectors

Th complete insert of pTCAD19 was excised by restriction of the plasmids with EcoR1. The inserts were made blunt ended and cloned into pJR1 and pMK4. The resulting vectors are called:

pJR1 based:

pJT19FS and pJT14FS (sense)

pJT19FA and pJT14FA (antisense)

pMK4 based:

pMT19FS and pMT14FS (sense)

pMT19FA and pMT14FA (antisense)

The construction of these vectors is shown in Figure 6.

Example 9

Transformation of tobacco using vectors described in this application

(a) Transfer of Vectors to Agrobacterium

The antisense and sense constructs were introduced into A.tumefaciens LBA4404 by direct transformation following published procedures.

The presence and integrity of the antisense constructs were checked by restriction digestion and Southern blot experiments to check that no recombination had occured during the transfer of the vectors to Agrobacterium.

b) Tobacco Leaf Disc Transformation

Tobacco (N. tabaccum, variety Samsum) leaf discs were transformed using well established previously published procedures. Plants containing the CAD antisense construct were identified by PCR and selected for further analysis.

Example 10

Analysis of transformed plants

a) CAD Enzyme Measur ments On Tissue From Transform d Plants

Plant material was used from both transformed and untransformed control plants for CAD enzyme determinations. Stem material was ground with CAD extraction buffer containing 200 mM Tris/HCl pH 7.5, 0.5% (w/v) PEG 6000, 5% (w/v) PVP, and 15 mM β-mercaptoethanol (500μl). The crude homogenate was centrifuged and the supernatant used as source of enzyme. The assay reaction contained 10 mM coniferyl alcohol (50μl), 10 mM NADP+ (50μl), 100 mM Tris/HCl pH 8.8 (800μl). This was incubated at 30°C for 10 minutes, then enzyme extract (100μl) was added and the whole mixture incubated for a further 10 minutes at 30°C. The OD400 was recorded against a blank supplemented with water. One sample was taken from each plant. Assays were conducted in duplicate. The results of the analysis of these enzyme measurement are shown in Figure 11. This clearly indicates that the transgenic plants exhibit a wide range of CAD enzyme activities. Plants with lowest levels of CAD activity show approximately 10% of control CAD enzyme values.

b) Polymerase Chain Reaction to determine presence of antisense genes

DNA was extracted from selected plants. Oligonucleotides to sequences in the CaMV or PAL promoter and nos 3′ terminator were used as primers in the polymerase chain reaction (PCR). To confirm that the products were CAD sequences, a Southern blot of these products was probed with a third oligonucleotide representing CAD sequences. This analysis demonstrated that all plants used for the analysis shown in Figure 10 contained the antisense

constructs.

Plants with low CAD activity were backcrossed and selfed. Progeny plants were analysed in detail for the heritability of the CAD antisense gene and the low CAD enzyme phenotype. This indicates that the low CAD phenotype segregates with the antisense gene.

Example 11

Isolation of a cDNA clone encoding poplar CAD.

80,000 colonies from a poplar cDNA library constructed in pUC18 were probed with the entire EcoR1 insert from pTCAD19. Hybridization was performed in 3xSSC,0.5% milk powder at 60°C. Washing was performed in 3xSSC, 0.5%SDS at 60°C for 3x 0.5 hour. One clone was isolated, named pPOPCAD1. This clone contained an insert of 1378bp and was 70% homologous to pTCAD19. The sequence of pPOPCAD1 is shown in Figure 7.

Example 12

Construction of sense/antisense vectors.

The insert of pPOPCAD1 was exised as a BamH1 fragment and cloned into the plant expression vector pGSJ780A in the antisense and sense orientations. These vectors were used to transform poplar and aspen.

Example 13

Establishment of a cDNA library from Eucalyptus

A cDNA library was generated using RNA extracted from 7 days old cell suspension cultures of Eucalyptus gunnii (clone 832, Afocel). 5μg of polyA+ RNA was prepared and used to synthesise cDNA. This was cloned into the EcoRI site of lambda gt11 (a commercially available cloning vector). This yielded 10^6 recombinants, 60% of which

have inserts of 1kb or greater, as determined by PCR on 24 randomly selected clones.

Example 14

Functional identification of the eucalyptus CAD clone

The identity of the eucalyptus CAD clone was confirmed by the expression of catalytically active CAD enzyme in a transformed E.coli bacterial host. This was achieved by cloning the eucalyptus CAD cDNA in the expression vector pT7-7 as described by Tabor and Richardson, Proceedings of the National Academy of Science 82, 1985, transforming the commercially available E.coli lysogenic strain BL21, inducing the expression of the cloned gene under control of the T7 promoter with IPTG and assaying the whole cell extract for CAD activity. The results unequivocally identified the clone as specifying the enzyme CAD.

Example 15

Cloning and characterization of a Eucalyptus CAD clone

600,00 recombinants from the amplified library (1.6 10^6 recombinants) were screened using the EcoRI insert of pTCAD19. Six positive clones were plaque purified; the largest has been subcloned into pGEM3 (a commercially available cloning vector), characterised and sequenced. This full length clone (1391 bp) encodes a protein of 356 amino acids which has a very high homology with the sequence of the tobacco CAD (76.4% of the amino acids are identical, 11% are well conserved).

The sequence of this clone, pEUCAD1 is shown in Figure 8.

Exampl 16

Cloning of a partial CAD clone from maize

PCR primers derived from sequences highly conserved between pTCAD19 and pPOPCAD1 (Figure 9) were used under suitable conditions to generate a PCR product from maize genomic DNA.The product was cloned into Bluescript SK+/- and its nucleotide sequence determined (Figure 10). This clone was clearly identified as encoding part of the maize CAD gene by DNA sequence comparison to the tobacco CAD sequences of pTCAD14/19.

Example 17

Analysis of cell wall bound and soluble phenolics in tobacco transformed with antisense tobacco CAD

CAD is believed to play a key role in the regulation of lignin biosynthesis and this Example reports confirmation of the effect of lignin down-regulation in transformants containing the antisense gene to CAD.

Lignin is known to react with thioglycolic acid (TGA) (Freudenberg et.al. in "Constitution and Biosynthesis of Lignin", Springer Verlag, Berlin, 1968) and methods employing TGA lignin extraction have been employed in the past to determine the amount of lignins present in plants after wounding. However, simple TGA extraction does tend to overestimate the amount of lignin because certain other components of the plant tissue are co-extracted. The simple method may be adapted to include a step of first saponifying the methanol insoluble component of the cell wall prior to TGA extraction (Campbell & Ellis, Phytochem 31: 737 (1992)).

Stem sections (5cm) from eight week old the

sample plants were lyohilised and separated into "green tissue" comprising the phloem, cort x and epidermis and "woody tissue" comprising the xylem and pith.

Ten samples each of control and transformed plants were analysed blind for TGA extractable cell wall complexes by the method described by Campbell and Ellis and also for the phenolics content of methanol and alkali extracts by the methods generally described by Ferraris et.al. J.Disease Protect. 94; 624 (1987).

The results are reported in Tables 1, 2, and 3 below.

TABLE 1

Direct numerical comparison of the content of TGA extractable complexes obtained from lyophilised stem tissue from control and transformed plants.

Tissue	Control Plants (C)	Transformed Plants (T)	Ratio T/C
green	0.03(0.01)	0.05(0.02)	1.53
woody	0.22(0.05)	0.37(0.12)	1.60
total	0.26(0.06)	0.42(0.13)	1.59

The units are A280/mg dry weight with the standard errors in parentheses

TABLE 2

Direct numerical comparison of the content of methanol-extractable phenolics obtained from lyophilised stem tissue from control and transformed plants.

Tissue	Control Plants(C)	Transformed Plants(T)	Ratio T/C
green	4.37(0.58)	5.64(0.76)	1.28
woody	0.81(0.12)	1.39(0.33)	1.72
total	5.18(0.65)	7.03(0.92)	1.35

The units are µg of ferulate equivalents per mg of dry weight with the standard errors shown in parentheses.

<u>TABLE 3</u>

<u>Direct numerical comparison of the content of alakali-extractable phenolics obtained from lyophilised stem tissue from control and transformed plants.</u>

Tissue	Control Plants(C)	Transformed Plants(T)	Ratio T/C
green	1.29(0.32)	2.13(0.52)	1.65
woody	0.82(0.17)	2.92(1.59)	3.58
total	2.10(0.46)	5.06(2.06)	2.4

The units are µg of ferulate equivalents per mg of dry weight with the standard errors shown in parentheses.

Although the results in Table 3 show an increase in the amount of TGA-extractable complexes in the CAD-antisense plants the increase can be explained by the suggestion that the chemical composition has altered and this would not entirely be surprising since the inhibition of CAD would inhibit synthesis of the type lignin polymers which are normally synthesised downstream of the CAD catalysed step in th pathway and result in a

build-up of the upstream phenolic acid precursors.

This change in the character of the lignin was confirmed by comparing the UV spectra of the TGA complexes from the control and transformed plants. Further confirmation has been obtained by alkaline nitrobenzene oxidation analysis which has revealed the presence of additional components in the transformed plants and chromatographic analysis indicates that these are phenolic acids.

Thus the TGA extraction analysis indicates that down-regulation of CAD makes the "lignin" more amenable to removal and this property should be reflected in facilitation of cellulose extraction processes.

The same feature is also indicated by the analyses reported in Tables 2 and 3. The alkaline extraction is further significant in that it is common farming practice to store forage crops as silage and this frequently involves addition of alkali (ammonia, usually) and it may be expected that silage made from forage crops tranformed with CAD antisense will have lower than normal lignin concentration, leading to improved digestibility.

CLAIMS

1. A recombinant DNA comprising a plant DNA having, in sequence, a gene promoter sequence, a coding region and a gene terminator, said coding region comprising a nucleotide sequence encoding a mRNA which is substantially homologous or complementary to mRNA encoded by an endogenous plant gene or a part thereof which encodes an enzyme essential to lignin biosynthesis, so that, when incorporated into a plant genome by transformation, mRNA transcribed from the said coding region inhibits production of the enzyme from the endogenous gene.

2. A recombinant DNA as claimed in claim 1 wherein the coding region encodes mRNA in antisense orientation to the mRNA encoded by the said endogenous gene.

3. A recombinant DNA as claimed in claim 2, in which the coding region is isolated from the untranscribed strand of the DNA encoding the said endogenous gene.

4. A recombinant DNA as claimed in claim 1 wherein the coding r gion is in the same orientation as the said ndogenous gene.

5. A r combinant DNA as claimed in any preceding claim in which the coding region has a minimum size of 50 bases.

6. A recombinant DNA as claimed in any preceding claim in which the said enzyme is selected from the group consisting of cinnamyl alcohol dehydrogenase (CAD), cinnamoyl: CoA reductase (CCR) and catechol-O-methyl transferase (COMT).

7. A recombinant DNA as claimed in any preceding claim in which the promoter is selected from the group consisting of CaMV35S, GPAL2, GPAL3 and endogenous plant promoter controlling expression of the endogenous CAD gene.

8. A method of inhibiting or altering lignin biosynthesis in a plant, comprising stably incorporating into the genome of the plant by transformation a recombinant DNA comprising a plant DNA having, in sequence a gene promoter sequence a coding region and a gene terminator, said coding region comprising a nucleotide sequence encoding a mRNA which is substantially homologous or complementary to mRNA encoded by an endogenous plant gene or a part thereof which encodes an enzyme essential to lignin biosynthesis, so that, when incorporated into a plant genome by transformation, mRNA transcribed from the said coding region inhibits production of the enzyme from the endogenous gene.

9. A transformed plant possessing lower than normal ability to produce lignin characterised in that said plant has stably incorporated within its genome a recombinant DNA claimed in any of claims 1 to 7.

10. A transformed plant, as claimed in claim 9, in which the plant species is alfalfa, maize, rape, eucalyptus, poplar, lolium or festuca.

11. Tobacco CAD gene and recombinant DNA containing same, derived from the plasmid pTCAD14 or pTCAD19 which have been deposited, in E.coli strain XL1Blue host, at the National Collection of Industrial and Marine Bacteria, Aberdeen, United Kingdom, under the Accession Number 40404 on 17th April 1991 and 40401 on 8th April 1991 respectively.

12. Maize CAD gene and recombinant DNA containing same, derived from the plasmid pZCAD1 which has been deposited, in E.coli strain XL1Blue host, on 2nd April 1992 at the National Collection of Industrial and Marine Bacteria, Aberdeen, United Kingdom, under the Accession Number 40501.

13. Poplar CAD gene and recombinant DNA containing same, derived from the plasmid pPOPCAD1 which has been deposited, in E.coli strain XL1Blue host, on 2nd April 1992 at the National Collection of Industrial and Marine Bacteria, Aberdeen, United Kingdom, under the Accession Number 40500.

14. Eucalyptus CAD gene and recombinant DNA containing same, derived from the plasmid pEUCAD1 which has been deposited, in E.coli strain XL1Blue host, on 2nd April 1992 at the National Collection of Industrial and Marine Bacteria, Aberdeen, United Kingdom, under the Accession Number 40502.

15. A recombinant DNA comprising an antisense CAD gene under control of the promoter GPAL2.

16. A recombinant DNA comprising an antisense CAD gene under control of the promoter GPAL3.

17. A recombinant DNA comprising an antisense CAD gene under control of the promoter CaMV35S.

18. A recombinant DNA comprising an antisense CAD gene under control of the endogenous plant promoter controlling the endogenous CAD gene.

FIG. 1 Peptide sequences obtained from tobacco CAD tryptic peptides.

	Peak 1 (44kDa)	Peak 2 (42 kDa)
N-TERMINAL SEQUENCE		
	K/SXLXV	K/SLXV
INTERNAL SEQUENCE		
1	TTIGXAAIVK	TAIGQAAIV
2	FPSDVLRPYTYTLD	PSGLLSPYTYTLV
3	FVVDVIGK	FVVDVAGD
4	MDYINGAMER	DYINTAMG/E
5	RTLGMSN	NDLGMSNYP
6	AMGXXVXVI	
7	AV/ITPYFD/Y	
8	SGILGL	

FIG. 2

Sequence of oligonucleotide used to identify a tobacco CAD clone

ATG GAT/C TAT/C ATT/C/A AAT/C GGI GCI ATG GA

FIG. 3 (1/2)

SEQ ID NO:1
SEQUENCE TYPE:Nucleotide
SEQUENCE LENGTH:1419 base pairs
STRANDEDNESS:single
TOPOLOGY:linear
MOLECULAR TYPE:cDNA

ORIGINAL SOURCE ORGANISM:tobacco var. Samsun
IMMEDIATE EXPERIMENTAL:tobacco stem cDNA library

FEATURES

from 93 - 1165 open reading frame
PROPERTIES: cDNA of cinnamyl alcohol dehydrogenase-pTCAD19

FIG. 3 (2/2)

```
ATTTCTTTCT CTTTCCCTTG AACTGTGTTT TCATTTTTTC TGCTCTGAAA CAATAGTGTT   60
TTCCTTGTAG ATTTTAAGTT AAAAGAAAAC CATGGGTAGC TTGGATGTTG AAAAATCAGC  120
TATTGGTTGG GCTGCTAGAG ACCCTTCTGG TCTACTTTCA CCTTATACCT ATACTCTCAG  180
AAACACAGGA CCTGAAGATG TGCAAGTCAA AGTTTTGTAT TGTGGACTTT GCCACAGTGA  240
TCTTCACCAA GTTAAAAATG ATCTTGGCAT GTCCAACTAC CCTCTGGTTC CTGGACATGA  300
AGTGGTGGGA AAAGTAGTGG AGGTAGGAGC AGATGTGTCA AAATTCAAAG TGGGGGACAC  360
AGTTGGAGTT GGATTACTCG TTGGAAGTTG TAGGAACTGT GGCCCTTGCA AGAGAGAAAT  420
AGAGCAATAT TGCAACAAGA AGATTTGGAA TTGCAATGAT GTCTACACTG ATGGCAAACC  480
CACCCAAGGT GGTTTTGCTA ATTCTATGGT TGTTGATCAA AACTTTGTGG TGAAAATTCC  540
AGAGGGTATG GCACCAGAAC AAGCAGCACC TCTATTATGT GCTGGCATAA CAGTATACAG  600
TCCATTCAAC CATTTTGGTT TTAATCAGAG TGGATTTAGA GGAGGAATTT TGGGATTAGG  660
AGGAGTTGGA CATATGGGAG TGAAAATAGC AAAGGCAATG GGACATCATG TTACTGTCAT  720
TAGTTCTTCA AATAAGAAGA GACAAGAGGC ATTGGAACAT CTTGGTGCAG ATGATTATCT  780
TGTTAGTTCA GACACTGATA AAATGCAAGA AGCTGCTGAT TCACTTGACT ATATTATTGA  840
TACTGTCCCT GTTGGCCATC CTCTTGAACT TTATCTTTCT TTGCTTAAAA TTGATGGCAA  900
ACTTATCTTG ATCGGAGTTA TCAACACCCC CTTGCAATTT ATCTCTCCCA TGGTTATGCT  960
CGGGAGAAAG AGCATCACTG GAAGCTTTAT TGGTAGCATG AAGGAAACAG AGGAAATGCT 1020
AGACTTCTGC AAAGAGAAAG GTGTGACTTC ACAGATTGAG ATAGTGAAAA TGGATTATAT 1080
CAACACTGCA ATGGAGAGGT TGGAGAAAAA TGATGTGAGC TACAGATTTG TTGTTGATGT 1140
TGCTGGAAGC AAGCTTGACC AGTAATTGCA CAAGAAAAAC AACATGGAAT GGTTCACTAT 1200
TATACAACAA GGCTATGAGA AAAATAGTAC TCCTCAACTT TGATGTCATC TTTGTTACCT 1260
TTGTTTTATT TTCCACCTGT ATTATCATAT TTGGTGGTCG AGAGTGACGT TTATGTATAT 1320
TTTCTTTCTT CAAAACAATC TTAAATGAAT TTGGATGTTG GTGACGATTT TGAAATATAC 1380
CAACCATGCA AACTTACTTT GGTAGAAAAA AAAAAAAAA                       1419
```

FIG. 4 (1 / 2)

SEQ ID NO: 2
SEQUENCE TYPE: Nucleotide
SEQUENCE LENGTH: 1393
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULAR TYPE: cDNA

ORIGINAL SOURCE ORGANISM: tobacco var. Samsum
IMMEDIATE EXPERIMENTAL: tobacco stem cDNA library

FEATURES:

from 84 - 1155 open reading frame

PROPERTIES: cDNA of cinnamyl alcohol dehydrogenase - pTCAD14

FIG. 4 (2/2)

```
TCTTTCCCTT GAACTGTGTT TTCGTTTTTT CTGCTCTAAA ACAATCGTGT GTTCCTTGTA   60
GATTTTAAGT TTAAAGAACA TCATGGGTGG CTTGGAAGTT GAGAAAACAA CTATTGGTTG  120
GGCTGCTAGA GACCCTTCTG GTGTACTTTC ACCTTATACC TATACTCTCA GAAACACAGG  180
ACCTGAAGAT GTGGAAGTCA AAGTTTTGTA TTGTGGGCTC TGTCACACTG ATCTTCACCA  240
AGTTAAAAAT GATCTTGGCA TGTCCAACTA CCCTCTGGTT CCTGGACATG AAGTGGTGGG  300
AGAAGTGGTG GAGGTAGGAC CAGATGTGTC AAAATTCAAA GTTGGGGACA CAGTTGGAGT  360
TGGATTACTC GTTGGAAGTT GCAGGAACTG TGGCCCTTGC AAGAGAGATA TAGAGCAATA  420
TTGCAACAAG AAGATTTGGA ACTGCAATGA TGTCTACACT GATGGCAAAC CCACCCAAGG  480
TGGTTTTGCT AAATCCATGG TTGTTGATCA AAAGTTTGTG GTGAAAATTC CAGAGGGTAT  540
GGCACCAGAA CAAGCAGCAC CTCTATTATG TGCTGGTATA ACAGTATACA GTCCATTGAA  600
CCATTTTGGT TTCAAACAGA GTGGATTAAG AGGAGGAATT TTGGGATTAG GAGGAGTGGG  660
ACACATGGGA GTGAAAATAG CAAAGGCAAT GGGACATCAT GTTACTGTCA TTAGTTCTTC  720
AAATAAGAAG AGACAAGAGG CATTGGAACA TCTTGGTGCA GATGATTATC TTGTCAGTTC  780
AGACACTGAT AAAATGCAAG AGGCTTCTGA TTCACTTGAC TATATTATTG ATACTGTCCC  840
TGTTGGCCAT CCTCTTGAAC CTTATCTTTC TTTGCTTAAA ATTGATGGCA AACTTATCTT  900
GATGGGAGTT ATCAACACCC CCTTGCAATT TATCTCCCCC ATGGTTATGC TCGGGAGAAA  960
GAGCATCACA GGAAGCTTTA TTGGTAGCAT GAAGGAAACA GAGGAAATGC TAGATTTCTG 1020
CAAAGAGAAA GGTGTGACTT CACAGATTGA GATAGTGAAA ATGGATTATA TCAACACTGC 1080
AATGGAGAGG TTGGAGAAAA ATGATGTGAG GTACAGATTT GTGGTTGATG TTATTGGAAG 1140
CAAGCTTGAC CAGTAATTAT ATTACACAAG AAAAACAACA TGGAATGGTT CACTATTATA 1200
CAAGGCTGTG AGAATACTAA ACTTTGATGT CGTCTTTTGT ATCCTTTTGT TTTATTTGCC 1260
ACCTGTATTT TCTTATTTGG TGATCGAGAG TGACGTTTAT GTATTATTTT CTTTCTTCAA 1320
AACAATTTAA TGTATGAATT TGGATGTTGG TGAAAAAAAA AAAAAAAAAA AAAAAAAAAA 1380
AAAAAAAAAA AAA                                                    1440
```

FIG. 5

CONSTRUCTION OF SENSE AND ANTISENSE VECTORS

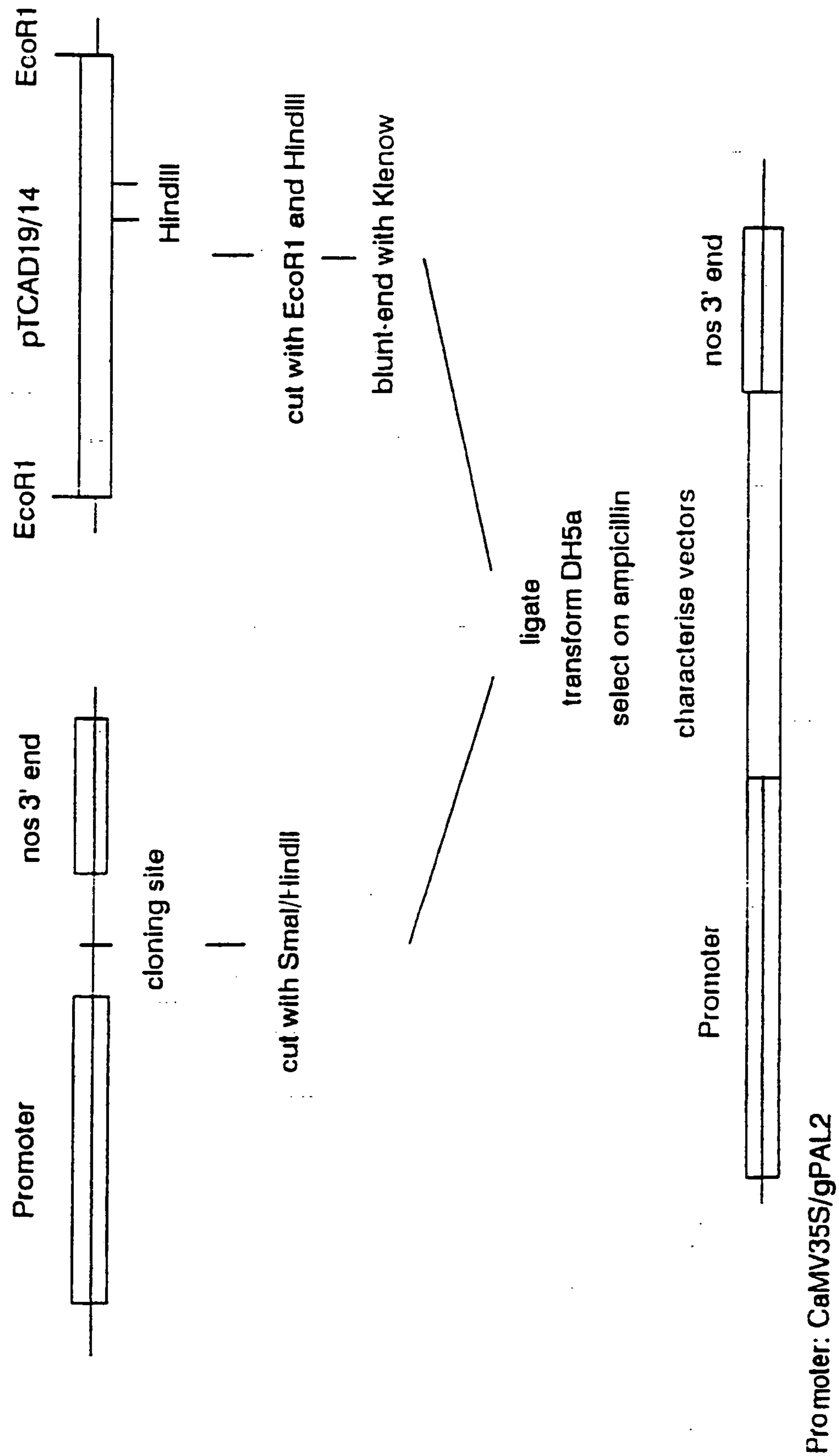
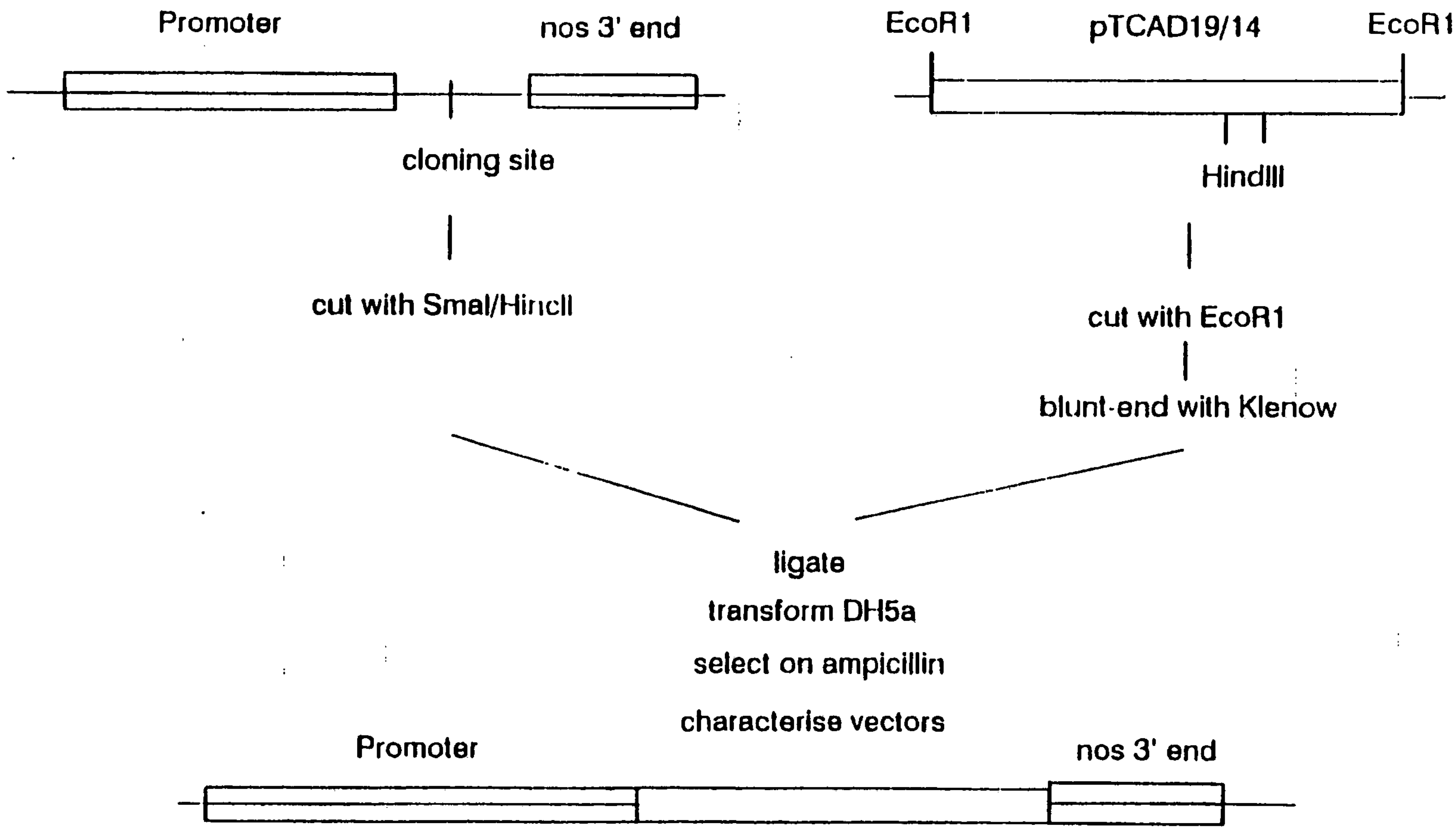


FIG.6

CONSTRUCTION OF SENSE AND ANTISENSE VECTORS



Promoter: CaMV35S/gPAL2

FIG.7 (1/2)

SEQ ID NO: 3
SEQUENCE TYPE: Nucleotide
SEQUENCE LENGTH: 1285
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULAR TYPE: cDNA

ORIGINAL SOURCE ORGANISM: poplar
IMMEDIATE EXPERIMENTAL: poplar xylem cDNA library

FEATURES:

from 28 - 1099 open reading frame

PROPERTIES: cDNA of cinnamyl alcohol dehydrogenase - pPOPCAD1

FIG. 7 (2 / 2)

```
CTCTCTTAGC CTCATTGTTT CAAGAAAATG GGTAGCCTTG AAACAGAGAG AAAAATTGTA   60
GGATGGGCAG CAACAGACTC AACTGGGCAT CTCGCTCCTT ACACCTATAG TCTCAGAGAT  120
ACGGGGCCAG AAGATGTTTT TATCAAGGTT ATCAGTTGTG GAGTTTGCCA TACCGATATC  180
CACCAAATCA AAAATGATCT TGGCATGTCA CACTATCCTA TGGTCCCTGG CCATGAAGTG  240
GTTGGTGAGG TTGTTGAGGT GGGATCAGAT GTGACAAGGT TCAAAGTTGG AGATGTTGTC  300
GGTGTTGGAG TCATCGTTGG AAGCTGCAAG AATTGTCATC CATGCAAATC AGAGATTGAG  360
CAATACTGCA ACAAGAAAAT CTGGTCTTAC AATGATGTCT ACACTGATGG CAAACCCACC  420
CAAGGAGGCT TTGCTGAATC CATGGTTGTG CATCAAAAGT TTGTGGTGAG AATTCCTGAT  480
GGGATGTCAC CAGAACAAGC AGCGCCGCTA TTGTGCGCTG GATTGACAGT TTACAGCCCA  540
CTTAAACACT TTGGACTGAA ACAGAGTGGG CTAAGAGGAG GGATTTTAGG ACTTGGAGGA  600
GTAGGGCACA TGGGGGTGAA GATAGCAAAG GCAATGGGAC ACCATGTAAC TGTGATTAGT  660
TCTTCTGACA AGAAGCGGGA GGAGGCTATG GAACATCTTG GTGCTGATGA ATACTTGGTC  720
AGCTCGGATG TGGAAAGCAT GCAAAAAGCT GCTGATCAAC TTGATTATAT CATCGATACT  780
GTGCCTGTGG TTCACCCTCT GGAGCCTTAC CTTTCTCTGT TGAAACTTGA TGGCAAGCTG  840
ATCTTGATGG GTGTTATTAA TGCCCCATTG CAGTTTGTTA CGCCTATGGT TATGCTTGGG  900
AGAAAGTCTA TCACCGGGAG CTTCATAGGG AGCATGAAGG AGACAGAGGA GATGCTTGAG  960
TTCTGCAAGG AAAAGGAGT GGCCTCCATG ATTGAAGTGA TCAAAATGGA TTATATCAAC1020
ACVGCATTCG AGAGGCTTGA GAAAAATGAT GTGAGATATA GATTCGTTGT CGATGTTGCT1080
GGTAGCAAGC TTATTCACTG AACAACAATA CTCTTCATAT TCGAAAAAAA AACGATATAC1140
ATTGATACCT GTTTCAGACG TGACTTTATT TCCGAGTGAT GTGTTTTGTG GATCAAATGT1200
GACAGTGTGT CTTTGCTTTT AAAATAAAGA AAAGGTTGAA TTGTTTTTTT NAAAAAAAAA1260
AAAAAAAAAA AAAAAAAAAA AAAAA                                        1320
```

FIG. 8 (1/2)

SEQ ID NO: 4
SEQUENCE TYPE: Nucleotide
SEQUENCE LENGTH: 1385
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULAR TYPE: cDNA

ORIGINAL SOURCE ORGANISM: eucalyptus gunnii (clone 832, AFOCEL)
IMMEDIATE EXPERIMENTAL: eucalyptus suspension culture cDNA library

FEATURES:

from 105 - 1173 open reading frame

PROPERTIES: cDNA of cinnamyl alcohol dehydrogenase - pEUCAD1

FIG.8 (2/2)

```
CTGCTCCTAC CCGCAACTTC CCATCTACAT AAGCAGCAAG TTTACGGCTC TGTCGAATCT   60
CTCTCCGAGC ACCACTTTGA AAGAAGCTTG GATCTTTGAG CAAAAATGGG CAGTCTTGAG  120
AAGGAGAGGA CCACCACGGG TTGGGCTGCA AGGGACCCGT CTGGCGTTCT CTCTCCTTAC  180
ACTTATAGCC TCAGAAACAC GGGACCAGAA GATCTTTACA TCAAGGTGTT GAGCTGCGGA  240
GTTTGCCACA GTGACATTCA CCAGATCAAG AATGATCTTG GCATGTCCCA CTACCCTATG  300
GTTCCTGGGC ATGAAGTGGT GGGCGAGGTT CTGGAGGTGG GATCAGAGGT GACAAAGTAC  360
AGAGTTGGTG ACCGAGTGGG AACCGGTATA GTGGTTGGGT GCTGCAGAAG CTGTAGCCCT  420
TGCAATTCGG ACCAGGAGCA ATATTGCAAC AAGAAGATTT GGAATTACAA TGACGTGTAC  480
ACCGATGGCA AGCCCACTCA AGGTGGGTTT GCTGGTGAGA TAGTGGTTGG CGAAAGGTTT  540
GTGGTGAAAA TCCCAGATGG GTTAGAGTCG GAACAGGCAG CGCCGCTGAT GTGCGCTGGT  600
GTGACCGTGT ACAGCCCTCT GGTGCGCTTT GGGCTCAAGC AAAGCGGGTT GAGAGGAGGG  660
ATATTGGGGC TTGGAGGGGT TGGCCACATG GGGGTGAAGA TAGCCAAGGC CATGGGACAC  720
CACGTGACTG TGATAAGCTC TTCTGATAAG AAGAGAACGG AGGCATTGGA GCACCTGGGT  780
GCCGATGCTT ACCTAGTGAG CTCCGATGAA AATGGAATGA AAGAGGCCAC TGATTCTCTC  840
GACTACATTT TTGACACTAT CCCTGTGGTT CACCCTCTCG AACCTTACCT GGCCTTGTTG  900
AAGCTCGATG GAAAGCTGAT CTTGACTGGT GTCATCAATG CTCCTCTTCA ATTTATCTCT  960
CCCATGGTTA TGCTTGGGAG GAAGTCAATC ACTGGGAGTT TCATAGGGAG CATGAAGGAA 1020
ACAGAGGAGA TGCTTGAGTT CTGCAAAGAA AAGGGATTGA CTTCCCAGAT CGAAGTGATC 1080
AAGATGGATT ATGTCAACAC CGCCCTAGAG AGGCTCGAGA AGAATGATGT CAGGTACAGG 1140
TTCGTCGTGG ACGTCGTGGG AAGCAAGCTT GATTAGTTTC GGCTTTCCCC ATAAGTAAAC 1200
AAGAAATCGA CTTGCTTGTC TCTCAATTCG AGTTCCTCAT GCCCTCTGTT GTATCATTGT 1260
TTGTTATACC GAGAGTGCTA TTTTCTTCTG TCTTCGTATT GAAACCATAG ACCTTCTCGA 1320
TTGTGTATTC AATGATGAAG GTGTTAATGA TTTTATCACT TAAAAAAAAA AAAAAAA    1380
```

FIG. 9

PCR primers used to isolate a maize CAD gene fragment

```
ZCAD3    CAT GAA GTG GTI GGI GAG GTI GTI GAG G
           C   G   C         A

ZCAD2    GGT TTI CCG TCI GTG TAC ACA TCA TTG
           C       A           G   G   G
```

FIG. 10

SEQ ID NO: 5
SEQUENCE TYPE: Nucleotide
SEQUENCE LENGTH: 180
STRANDEDNESS: single
TOPOLOGY: linear
MOLECULAR TYPE: genomic fragment

ORIGINAL SOURCE ORGANISM: maize
IMMEDIATE EXPERIMENTAL: pcr product from genomic DNA

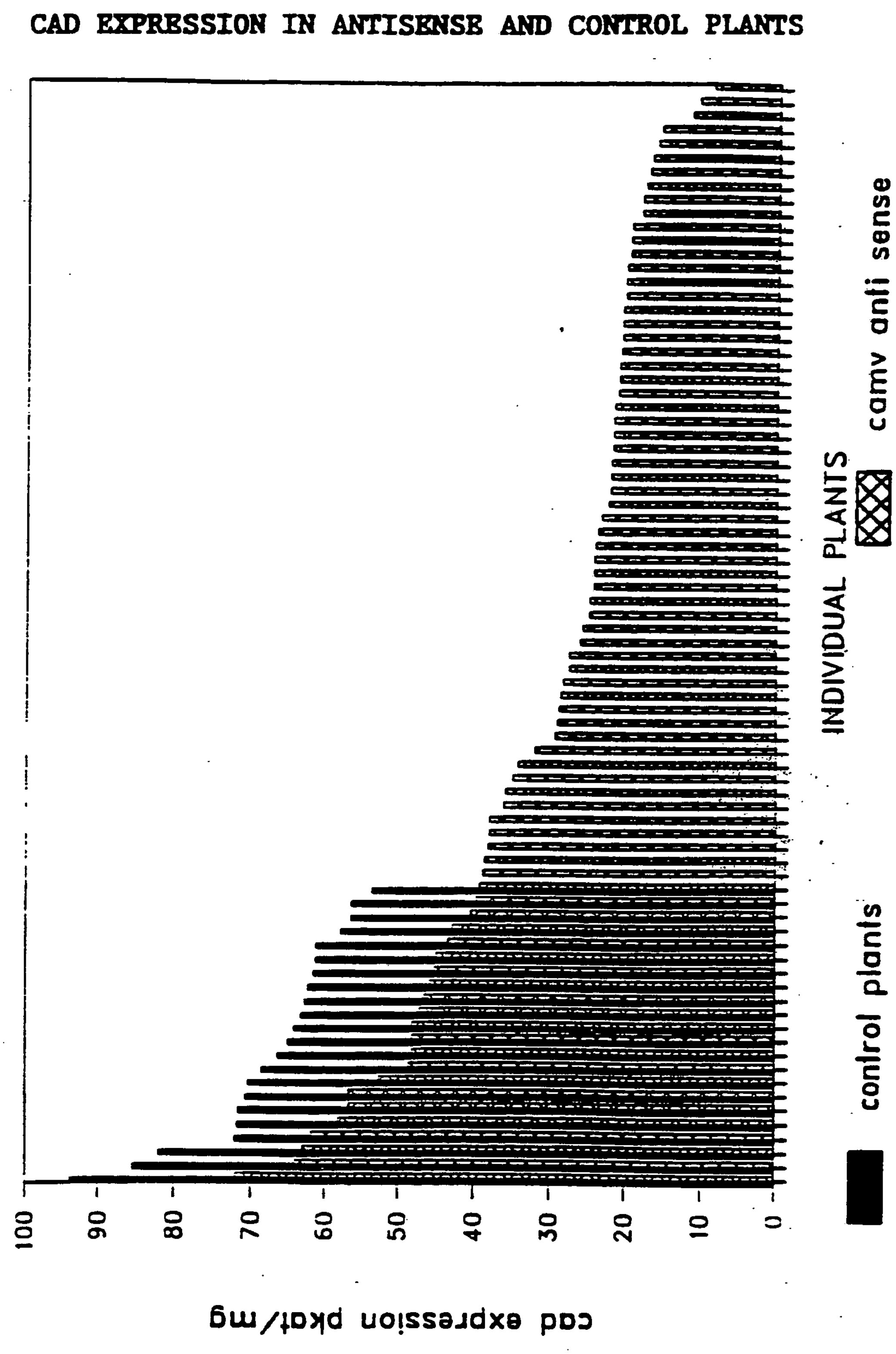
FEATURES:

from 1-180 open reading frame

PROPERTIES: genomic fragment of cinnamyl alcohol dehydrogenase - pZCAD1

```
GGTGGTGGGG GAGGTGGTGG AGGTCGGGCC CGAGGTGGCC AAGTACGGCT TCGGCGACGT  60
GGTAGGCGTC GGGGTGATCG TTGGGTCGTG CCGCGAGTGC AGCCCCTGCA AGGCCAACGT 120
TGAGCAGTAC TGCAACAAGA AGATCTGGTC ATACAACGAC GTCTACACCG ACGGCAAACC 180
```

FIG.11


CAD EXPRESSION IN ANTISENSE AND CONTROL PLANTS
cad expression pkat/mg
100
90
80
70
60
50
40
30
20
10
0
INDIVIDUAL PLANTS
control plants
camv anti sense

PCT/GB 92/00774

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

REC'D 18 MAY 1992
WIPO PCT

Imperial Chemical Industries plc,
ICI Seeds,
Jealott's Hill Research Station,
Bracknell,
Berkshire.
RG12 6EY

NAME AND ADDRESS OF DEPOSITOR

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT issued pursuant to Rule 7.1 by the INTERNATIONAL DEPOSITARY AUTHORITY identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
Escherichia coli XL1-Blue pZCAD1	NCIMB 40501

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

[ ] a scientific description

[X] a proposed taxonomic designation

(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on 2 April 1992 (date of the original deposit)[1]

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on (date of the original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion)

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: NCIMB Ltd

Address: 23 St Machar Drive, Aberdeen, Scotland, UK AB2 1RY.

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):

Date: 3 April 1992

1 Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired.

Form BP/4 (sole page)

PCT/GB 92/00774

18 MAY 1992

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

Mr. F.M. Huskisson,
Chartered Patent Attorney,
Imperial Chemical Industries plc,
ICI Seeds,
Jealott's Hill Research Station,
Bracknell, Berkshire.
RG12 6EY

NAME AND ADDRESS OF THE PARTY
TO WHOM THE VIABILITY STATEMENT
IS ISSUED

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified on the following page

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Imperial Chemical Industries plc, ICI Seeds, Address: Jealott's Hill Research Station, Bracknell, Berkshire. RG12 6EY	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: NCIMB 40501 Date of the deposit or of the transfer:[1] 2 April 1992

III. VIABILITY STATEMENT

The viability of the microorganism identified under II above was tested on 2 April 1992 [2]. On that date, the said microorganism was

[X] [3] viable

[ ] [3] no longer viable

1 Indicate the date of the original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

2 In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent viability test.

3 Mark with a cross the applicable box.

Form BP/9 (first page)

1 8 MAY 1992

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED[4]

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: NCIMB Ltd Address: 23 St Machar [illegible] Aberdeen [illegible] UK [illegible]	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Date: 3 April 1992

[4] Fill in if the information has been requested and if the results of the test were negative.

Form BP/9 (second and last page)

PCT/GB 92/00/74

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

18 MAY 1992

Imperial Chemical Industries plc,
ICI Seeds,
Jealott's Hill Research Station,
Bracknell,
Berkshire.
RG12 6EY

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT issued pursuant to Rule 7.1 by the INTERNATIONAL DEPOSITARY AUTHORITY identified at the bottom of this page

NAME AND ADDRESS OF DEPOSITOR

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
Escherichia coli XL1-Blue pPOPCAD1	NCIMB 40500

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

[ ] a scientific description

[X] a proposed taxonomic designation

(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on 2 April 1992 (date of the original deposit)[1]

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on (date of the original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion)

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: NCIMB Ltd	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):
Address: 23 St Machar Drive, Aberdeen, Scotland, UK AB2 1RY	Date: 3 April 1992

1 Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired.

Form BP/4 (sole page)

1 8 MAY 1992

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

Mr. F.M. Huskisson,
Chartered Patent Attorney,
Imperial Chemical Industries plc,
ICI Seeds,
Jealott's Hill Research Station,
Bracknell, Berkshire.
RG12 6EY

NAME AND ADDRESS OF THE PARTY TO WHOM THE VIABILITY STATEMENT IS ISSUED

INTERNATIONAL FORM

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified on the following page

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Imperial Chemical Industries plc, ICI Seeds, Address: Jealott's Hill Research Station, Bracknell, Berkshire. RG12 6EY	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: NCIMB 40500 Date of the deposit or of the transfer:[1] 2 April 1992

III. VIABILITY STATEMENT

The viability of the microorganism identified under II above was tested on 2 April 1992[2]. On that date, the said microorganism was

[X][3] viable

[ ][3] no longer viable

1 Indicate the date of the original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

2 In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent viability test.

3 Mark with a cross the applicable box.

Form BP/9 (first page)

18 MAY 1992

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED[4]

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: NCIMB Ltd Address: 23 St Machar Drive Aberdeen Scotland UK AB2 1RY	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Date: 3 April 1992

4 Fill in if the information has been requested and if the results of the test were negative.

PCT/GB 92/00774

18 MAY 1992

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

Imperial Chemical Industries plc,
ICI Seeds,
Jealott's Hill Research Station,
Bracknell,
Berkshire.
RG12 6EY

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT issued pursuant to Rule 7.1 by the INTERNATIONAL DEPOSITARY AUTHORITY identified at the bottom of this page

NAME AND ADDRESS OF DEPOSITOR

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
Escherichia coli DH5α pEUCAD1	NCIMB 40502

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

[ ] a scientific description

[X] a proposed taxonomic designation

(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on 2 April 1992 (date of the original deposit)[1]

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on (date of the original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion)

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: NCIMB Ltd	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorised official(s):
Address: 23 St Machar Drive Aberdeen Scotland UK AB2 1RY	Date: 3 April 1992

1 Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired.

Form BP/4 (sole page)

PCT/GB 92/00774

18 MAY 1992

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

Mr. F.M. Huskisson,
Chartered Patent Attorney,
Imperial Chemical Industries plc,
ICI Seeds,
Jealott's Hill Research Station,
Bracknell, Berkshire.
RG12 6EY

INTERNATIONAL FORM

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified on the following page

NAME AND ADDRESS OF THE PARTY
TO WHOM THE VIABILITY STATEMENT
IS ISSUED

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Imperial Chemical Industries plc, ICI Seeds, Address: Jealott's Hill Research Station, Bracknell, Berkshire. RG12 6EY	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: NCIMB 40502 Date of the deposit or of the transfer:[1] 2 April 1992

III. VIABILITY STATEMENT

The viability of the microorganism identified under II above was tested on 2 April 1992 [2]. On that date, the said microorganism was

[X][3] viable

[ ][3] no longer viable

1 Indicate the date of the original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

2 In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent viability test.

3 Mark with a cross the applicable box.

Form BP/9 (first page)

1 8 MAY 1992

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED[4]

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: Address: NCIMB Ltd 23 St Machar Drive Aberdeen	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Date: 3 April 1992

4 Fill in if the information has been requested and if the results of the test were negative.

Form BP/9 (second and last page)

PCT/GB 92/00774

18 MAY 1992

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANIS...
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

TO

Imperial Chemical Industries plc,
ICI Seeds,
Jealotts Hill Research Station,
Bracknell, Berkshire. RG12 6EY

NAME AND ADDRESS OF DEPOSITOR

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
Escherichia coli XL1-Blue pTCAD14	NCIMB 40404

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

[ ] a scientific description

[X] a proposed taxonomic designation

(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on 17 April 1991 (date of the original deposit)[1]

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on (date of the original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion)

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: **NCIMB Ltd**

Address: 23 St Machar Drive
Aberdeen Scotland
UK AB2 1RY

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):

Date: 19 April 1991

1 Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired.

Form BP/4 (sole page)

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

Mr. F.M. Huskisson,
Imperial Chemical Industries plc,
ICI Seeds,
Jealotts Hill Research Station,
Bracknell,
Berkshire. RG12 6EY

NAME AND ADDRESS OF THE PARTY
TO WHOM THE VIABILITY STATEMENT
IS ISSUED

INTERNATIONAL FORM

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified on the following page

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Imperial Chemical Industries plc. Address: AS ABOVE	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: NCIMB 40404 Date of the deposit or of the transfer:[1] 17 April 1991

III. VIABILITY STATEMENT

The viability of the microorganism identified under II above was tested on 17 April 1991[2]. On that date, the said microorganism was

[X][3] viable

[ ][3] no longer viable

1 Indicate the date of the original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

2 In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent viability test.

3 Mark with a cross the applicable box.

Form BP/9 (first page)

PCT/GB 92/00774
18 MAY 1992

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED[4]	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: NCIMB Ltd Address: 23 St Machar Drive Aberdeen Scotland UK AB2 1RY	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Date: 19 April 1991

4 Fill in if the information has been requested and if the results of the test were negative.

PCT/GB 92/00077
18 MAY 1992

BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

TO Imperial Chemical Industries plc
ICI Seeds
Jealotts Hill Research Station
Bracknell, Berkshire
RG12 6EY

NAME AND ADDRESS OF DEPOSITOR

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT issued pursuant to Rule 7.1 by the INTERNATIONAL DEPOSITARY AUTHORITY identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
Escherichia coli XL1-Blue pTCAD19	NCIMB 40401

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

[ ] a scientific description

[X] a proposed taxonomic designation

(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on 8 April 1991 (date of the original deposit)[1]

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on (date of the original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion)

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: NCIMB Ltd
23 St Machar Drive
Aberdeen Scotland
Address: UK AB2 1RY

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):

Date: 11 April 1991

1 Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired.

Form BP/4 (sole page)

18 MAY 1992

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

Mr F M Huskisson
Imperial Chemical Industries plc
ICI Seeds
Jealotts Hill Research Station
Bracknell, Berkshire RG12 6EY

NAME AND ADDRESS OF THE PARTY
TO WHOM THE VIABILITY STATEMENT
IS ISSUED

INTERNATIONAL FORM

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified on the following page

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Imperial Chemical Industries plc Address: ICI Seeds Jealotts Hill Research Station Bracknell, Berkshire RG12 6EY	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: NCIMB 40401 Date of the deposit or of the transfer: 8 April 1991

III. VIABILITY STATEMENT

The viability of the microorganism identified under II above was tested on 8 April 1991 [2]. On that date, the said microorganism was

[X] [3] viable

[ ] [3] no longer viable

1 Indicate the date of the original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

2 In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent viability test.

3 Mark with a cross the applicable box.

08 MAY 1992

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED[4]	
V. INTERNATIONAL DEPOSITARY AUTHORITY	
Name: **NCIMB Ltd** Address: **23 St Machar Drive** **Aberdeen Scotland** **UK AB2 1RY**	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Date: 11 April 1991

[4] Fill in if the information has been requested and if the results of the test were negative.

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 92/00774

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/53; C12N15/82; A01H5/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; A01H

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category°	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	CHEMICAL ABSTRACTS, vol. 114, 1991, Columbus, Ohio, US; abstract no. 222827, SCHUCH, W. W., ET AL.: 'Transgenic plants having reduced lignin or lignin of altered quality' see abstract	1-10, 15-18
Y	& CA,A,2 005 597 (ICI) 15 June 1990	6,11-14
O,Y	J. CELL. BIOCHEM. SUPPL. vol. 14E, 1990, page 355; O'MALLEY, D.M.,ET AL.: 'Purification and characterizatton of cinnamyl alcohol dehydrogenase from developing xylem of loblolly pine, and its role in stategies to modify the lignin content of wood' see abstract R532	11-14

-/--

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
03 SEPTEMBER 1992	11. 09. 92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	MADDOX A.D.

2

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	EP,A,0 240 208 (CALGENE) 7 October 1987 see page 4, line 38 - line 40 ---	6
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 87, November 1990, WASHINGTON US pages 9057 - 9061; ELKIND, Y., ET AL.: 'Abnormal plant development and down-regulation of phenylpropanoid biosynthesis in transgenic tobacco containing a heterologous phenylalanine ammonia-lyase gene' see page 9058, line 17 - line 26 ---	1,4,5, 8-10
O,X	UCLA SYMP. MOL. CELL. BIOL., NEW SER., PLANT GENE TRANSFER SYMPOSIUM HELD APRIL1-7 1989 vol. 129, 1990, pages 135 - 141; ROTHSTEIN, S. J., ET AL.: 'Inhibition of nopaline synthase and peroxidase expression in tobacco expressing antisense RNA' see page 140, line 15 - line 19 ---	1,8
A	PLANT MOLECULAR BIOLOGY. vol. 15, 1990, DORDRECHT, THE NETHERLANDS. pages 525 - 526; WALTER, M. H., ET AL.: 'Extensive sequence similarity of the bean CAD4 (cinnamyl-alcohol dehydrogenase) to a maize malic enzyme' see the whole document ---	1-18
O,A	J. CELL. BIOCHEM. SUPPL. vol. 14E, 1990, page 353; LOOPSTRA, C. A., ET AL.: 'Isolation of genes with enhanced expression in xylem tissue of loblolly pine' see abstract R525 ---	1-18
O,A	J. CELL. BIOCHEM. SUPPL. vol. 14E, 1990, page 348; GRIMA-PETTENATI, J., ET AL.: 'Inhibition of cinnamyl alcohol dehydrogenase synthesis in stably transformed plants expressing antisense RNA' see abstract R511 ---	1-18

-/--

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,A	PLANT PHYSIOLOGY. vol. 98, no. 1, January 1992, ROCKVILLE, MD, USA. pages 12 - 16; HALPIN, C.,ET AL.: 'Purification and characterization of cinnamyl alcohol dehydrogenase from tobacco stems' see the whole document	1-18
A	Biological abstracts vol.78 ref. no. 14461 see the abstract & EUR. J. BIOCHEM. vol. 139, no. 2, 1984, pages 259 - 266; SARNI, F., ET AL.: 'Purification and properties of cinnamoyl-coenzyme A reductase (EC 1.2.1.44) and cinnamyl alcohol dehydrogenase (EC 1.1.1.1.) from poplar stems (Populus euramericana)'	13
P,A	PLANT PHYSIOLOGY. vol. 98, no. 4, April 1992, ROCKVILLE, MD, USA. pages 1364 - 1371; O'MALLEY, D. M., ET AL.: 'Purification, chracterization, and cloning of cinnamyl alcohol dehydrogenase in loblolly pine (Pinus taeda L.)' see the whole document	13

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 9200774 SA 58858

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 03/09/92
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
CA-A-2005597	15-06-90	None	
EP-A-0240208	07-10-87	AU-B- 618234	19-12-91
		AU-A- 7059787	01-10-87
		EP-A- 0458367	27-11-91
		JP-A- 62296880	24-12-87
		US-A- 5107065	21-04-92
		US-A- 4801540	31-01-89

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82